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Chemical Suppression of Steroidogenesis

by Howard D. Colby*

A large number of chemicals are known to interfere with steroidogenesis in the adrenal cortex and other tissues. Many xenobiotics inhibit steroid hormone production as a result of interactions with cytochrome P-450-containing hydroxylases in adrenal mitochondria or microsomes. For example, metyrapone, a compound used clinically in the evaluation of pituitary-adrenocortical function, binds to various cytochromes P-450 in the adrenal, preventing the interactions of steroid substrates with the enzymes and inhibiting steroidogenesis. The mineralocorticoid antagonist, spironolactone, and its major circulating metabolite, canrenone, also competitively interact with adrenal steroid hydroxylases. In addition, spironolactone is converted by adrenal microsomes to an unknown metabolite which promotes the destruction of cytochromes P-450, decreasing the activities of steroid hydroxylases. Carbon tetrachloride is similarly "activated" by adrenal microsomal mixed function oxidases resulting in a decline in steroidogenic enzyme activity. Carbon tetrachloride (in the presence of NADPH) initiates lipid peroxidation in adrenal microsomes but its toxic effects on steroid hydroxylases are fully demonstrable when lipid peroxidation is inhibited by EDTA. A number of heavy metals, including cadmium, also inhibit adrenal steroid hydroxylases. When incubated with adrenal microsomes, cadmium does not affect cytochrome P-450 levels but decreases basal and substrate stimulated NADPH-cytochrome P-450 reductase activity. Although inhibitory effects of many chemicals on steroidogenesis have been described, the toxicological significance as well as definitive mechanisms of action have in most cases yet to be determined.

There are numerous substances which are known to interfere with the production of steroid hormones. The types of compounds range from therapeutic and diagnostic agents to environmental toxins and chemical carcinogens. In addressing the topic of chemical suppression of steroidogenesis I shall not attempt to catalog all of those compounds. Instead, this presentation will concentrate on a few different classes of chemical agents with the objective of illustrating several mechanisms through which chemicals can affect steroid hormone production. For the sake of simplicity and uniformity, only the effects of chemicals on steroidogenesis in the adrenal cortex will be discussed although in many cases similar effects are also manifested in other steroid-producing tissues. Furthermore, in keeping with the theme of this conference, greater emphasis will be placed on those compounds whose effects on steroidogenesis are more in the realm of toxicology than on those drugs which have been developed specifically to inhibit steroidogenesis.

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Before discussing the actions of chemicals on steroidogenesis, it is important to introduce some background material on the types of enzymatic reactions involved in the production of steroid hormones so that the actions of chemicals on these enzymes can be more fully appreciated. Figure 1 illustrates a portion of the steroidogenic pathway which exists in the adrenal cortex. Some of the pathway overlaps with that in other steroidogenic tissues. However, production of glucocorticoids, such as cortisol, and of mineralocorticoids, like aldosterone, is specific for the adrenal. Of particular interest are the role of cholesterol as the common precursor to all steroid hormones and the number of hydroxylation reactions involved in the steroidogenic pathway. For example, note the 11β, 18, 17α, and 21-hydroxylases as well as the cholesterol sidechain cleavage reaction which includes 2 hydroxylation steps (*1*, *2*). Thus, hydroxylases are critically important in steroidogenesis and as will be seen, represent the sites of action of many chemicals which influence steroidogenesis.

The adrenal steroid hydroxylases are typical mixed function oxidases which contain the heme-

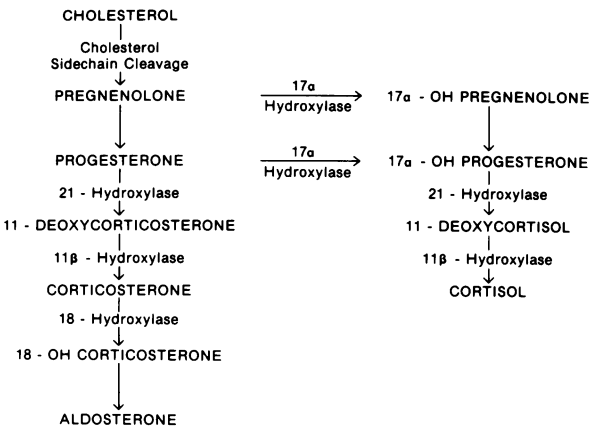


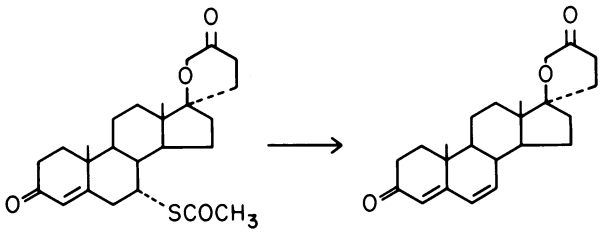
FIGURE 1. Pathways for the production of glucocorticoids and mineralocorticoids in the adrenal cortex.

protein, cytochrome P-450. They are similar to those hepatic enzymes involved in the oxidative metabolism of drugs, carcinogens and other xenobiotics (*3*, *4*). All require NADPH and molecular O_2 as cofactors and are membrane-bound enzymes. Some are located in mitochondria and others in the endoplasmic reticulum of the cell (*1-4*). The mitochondrial and microsomal enzymes differ somewhat in their composition but for the purpose of this discussion, all can be thought of as two functional units, the cytochrome P-450 to which the steroid substrate binds, and a reductase component which provides electrons derived from NADPH to the cytochrome P-450. Once the substrate binds to the cytochrome P-450, the reductase provides electrons to reduce the substrate-cytochrome complex and also to promote activation of oxygen for incorporation into the steroid nucleus. The specific site of hydroxylation of the steroid as well as the substrate specificity of the enzyme is governed by the cytochrome P-450 component. The number of different cytochromes P-450 involved in steroidogenesis has yet to be determined, but it is possible, perhaps even likely, that each hydroxylase contains its own distinct cytochrome P-450 moiety. Both the reductase and cytochrome P-450 components of the enzyme are essential for hydroxylase activity.

A large number of the chemicals which have been employed clinically as inhibitors of steroidogenesis interact with various cytochromes P-450 in steroid-producing tissues and inhibit the binding of normal steroid substrates, thereby decreasing hydroxylase activities (*5*). One of the best known and most thoroughly studied of this group of compounds is the pyridine derivative, metyrapone (SU-4885), which is widely used in the evaluation of pituitary-adrenocortical function. By inhibiting cortisol production, metyrapone should elicit an increase in pituitary ACTH secretion and thereby serves as a test of pituitary secretory reserve (*5*).

When metyrapone is added to adrenal mitochondrial preparations, it produces a characteristic difference spectrum, indicative of its binding to cytochromes P-450 (*6*). The spectrum is characterized by an increase in absorbance at about 425 nm and a decrease at about 400 nm and is known as a type II spectral change. The size of the spectrum increases with increasing concentrations of metyrapone until its binding sites on the enzyme are saturated. In the presence of metyrapone the size of the spectral change resulting from substrate interactions with adrenal mitochondrial cytochromes P-450 is diminished (*5*, *6*), illustrating the mechanism of action of the drug on steroid hydroxylation reactions.

Although originally proposed to be a relatively specific 11β-hydroxylase antagonist, metyrapone has been found to inhibit various other steroid hydroxylation reactions, including 18- and 19-hydroxylation and cholesterol sidechain cleavage (*5*, *7*). Each of these enzymes is located in mitochondria and the results of several early studies suggested that adrenal microsomal hydroxylases were not affected by metyrapone. Neither 21-hydroxylase nor 17α-hydroxylase activity was inhibited by metyrapone in rat or bovine adrenals (*8*, *9*). However, the specificity of the actions of metyrapone appears to be species-dependent (*10*). In the rat adrenal (Fig. 2), for example, concentrations of metyrapone which produced inhibition of mitochondrial 11β-hydroxylation had no effects on microsomal 21-hydroxylase activity. In the guinea pig (Fig. 3), by contrast, metyrapone had similar effects on 11β-and 21-hydroxylation. Observations such as these indicate that care must be taken when using compounds like metryapone for experimental purposes since the specificity of their actions is not as great as some earlier studies suggested and is influenced by a number of variables.



Spironolactone Canrenone

Another compound which is used clinically and which has been shown to affect steroidogenesis is

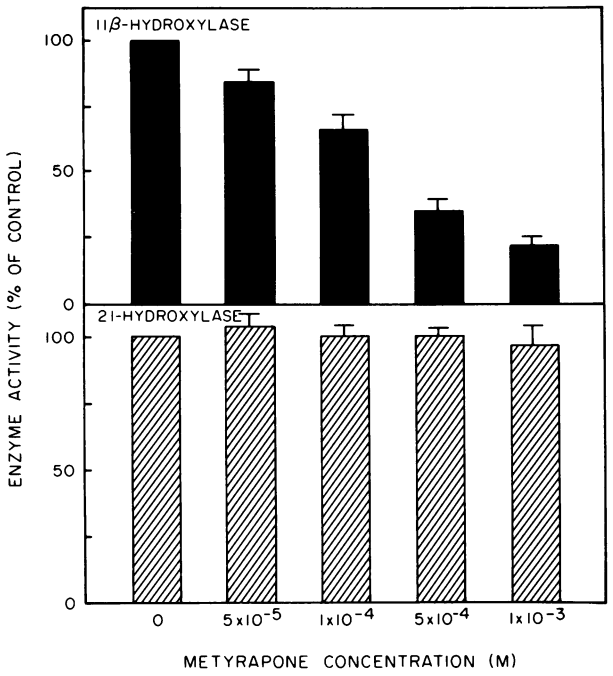


FIGURE 2. Effects of metyrapone on 11β- and 21-hydroxylase activities in rat adrenal mitochondria and microsomes, respectively. See Greiner et al. (*10*) for experimental details.

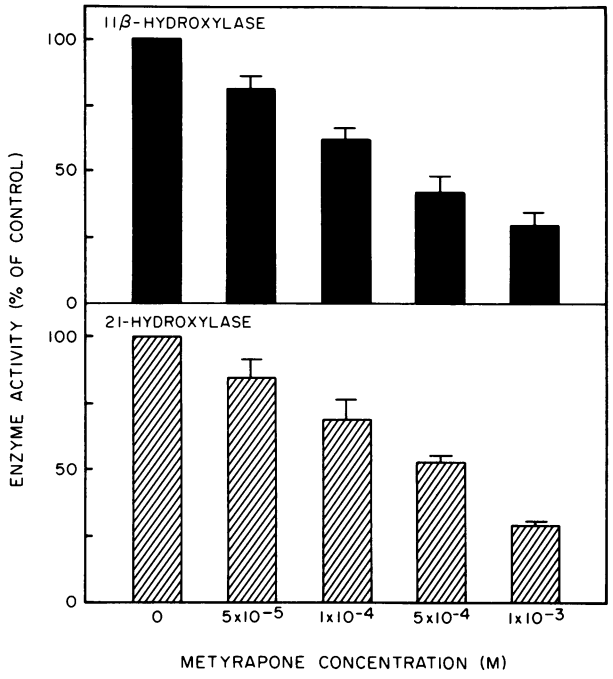


FIGURE 3. Effects of metyrapone on 11β- and 21-hydroxylase activities in guinea pig adrenal mitochondria and microsomes, respectively. See Greiner et al. (*10*) for experimental details.

Table 1. Effects of spironolactone administration to guinea pigs on cortisol production by adrenal slices.[a]

	Control	Spironolactone
Body weight, g	657 + 12	635 ± 10
Adrenal weight, mg/pair	340 ± 17	358 ± 10
Cortisol production by adrenal slices, nmoles/100mg/hr		
-ACTH	31.2 ± 3.9	13.2 ± 2.2[b]
+ACTH	64.7 ± 7.2	29.5 ± 4.1[b]

[a] For experimental details, see Greiner et al. (*15*).
[b] $p < 0.05$ (vs. controls).

the mineralocorticoid antagonist, spironolactone (SL). SL is used as a diuretic and in the treatment of some types of hypertension. Several clinical observations indicated that SL might interfere with adrenal steroidogenesis in man (*11*, *12*) and prompted a number of studies on its effects in experimental animals (*13-17*).

Following the administration of SL (25 mg/kg/day for 3 days) to guinea pigs there was a decline in cortisol production by adrenal sections *in vitro* (Table 1) (*15*). The deficit was apparent whether or not the adrenals were incubated with ACTH to stimulate steroidogenesis. Indirect effects of SL on the adrenal cortex resulting from inhibition of ACTH secretion were excluded by comparing the actions of SL with those of cortisone, a potent inhibitor of pituitary ACTH secretion (*15*). Once the inhibitory effect of SL on adrenocortical function was established, further studies were carried out to determine its mechanism of action as well as the role of canrenone, the major circulating metabolite of SL, in the actions of SL on the adrenal.

Either SL or conrenone administration to guinea pigs decreased the activities of several steroid hydroxylases in both adrenal mitochondria and microsomes (Table 2) (*16*). The effects of SL on the microsomal enzymes were greater than those of canrenone. The decline in steroidogenic activity could be attributed in part to the direct interactions of SL and canrenone with steroid hydroxylases. Both compounds produced type I difference spectra in adrenal mitochondria and microsomes, indicative of binding to cytochrome(s) P-450, and interfered with the binding of steroid substrates to cytochromes P-450 (*16*). As a result, SL and canrenone were direct inhibitors of mitochondrial and microsomal steroid hydroxylation *in vitro* (Figs. 4 and 5). Each compound produced a concentration-dependent inhibition of mitochondrial 11β-hydroxylation and microsomal 21-hydroxylation. The effects of canrenone on both enzymes were greater than those of SL. Thus, the relative potencies *in vitro* were opposite those obtained after *in vivo* administration of SL

Table 2. Effects of spironolactone or canrenone administration to guinea pigs on adrenal steroid hydroxylases.[a]

	Enzyme activities, nmole/min/mg protein		
	Control	Spironolactone	Canrenone
Mitochondrial			
11β-Hydroxylase	2.4 ± 0.1	1.5 ± 0.1[b]	1.7 ± 0.1[b]
Cholesterol SCC	0.28 ± 0.03	0.15 ± 0.02[b]	0.17 ± 0.02[b]
Microsomal			
17α-Hydroxylase	6.9 ± 0.7	2.3 ± 0.3[b]	4.5 ± 0.5[bc]
21-Hydroxylase	4.4 ± 0.3	1.6 ± 0.2[b]	2.9 ± 0.1[bc]

[a] For experimental details, see Greiner et al. (*16*).
[b] $p < 0.05$ (vs. controls)
[c] $p < 0.05$ (vs. spironolactone-treated)

and canrenone (Table 2). However, direct competition for binding to cytochromes P-450 provided only part of the explanation for the actions of SL on steroidogenesis. SL treatment also resulted in a decline in the amount of cytochromes P-450 in adrenal mitochondria and microsomes (Table 3). Canrenone, in contrast, had no effect on cytochrome P-450 concentrations, which may account for the greater potency of SL *in vivo* on steroidogenic enzymes.

The effect of SL on adrenal cytochrome P-450 levels could also be demonstrated *in vitro* under the appropriate experimental conditions (*14*, *16*). When adrenal microsomes were incubated with SL plus NADPH, a decline in cytochrome P-450 levels resulted (Fig. 6). Omission of either SL or NADPH from the incubation medium eliminated the effect. In contrast, incubation with canrenone, with or without NADPH, did not affect cytochrome P-450 content. The decline in cytochrome P-450 levels caused by SL plus NADPH was accompanied by a fall in microsomal heme content. The specificity of action of SL on cytochrome P-450 is indicated by the absence of effects on the other major adrenal microsomal hemeprotein, cytochrome b_5 (Fig. 6). These and other observations indicate that SL is

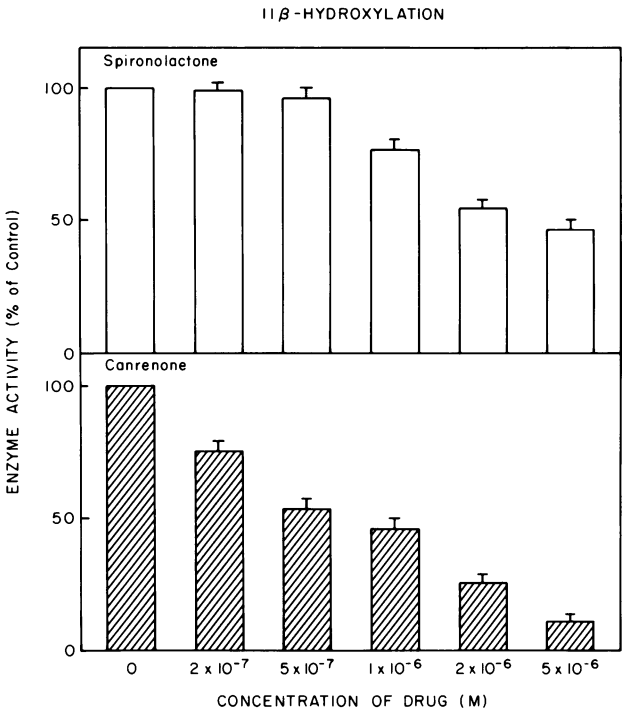


FIGURE 4. Effects of spironolactone and canrenone *in vitro* on 11β-hydroxylase activity in guinea pig adrenal mitochondria. See Greiner et al. (*16*) for experimental details.

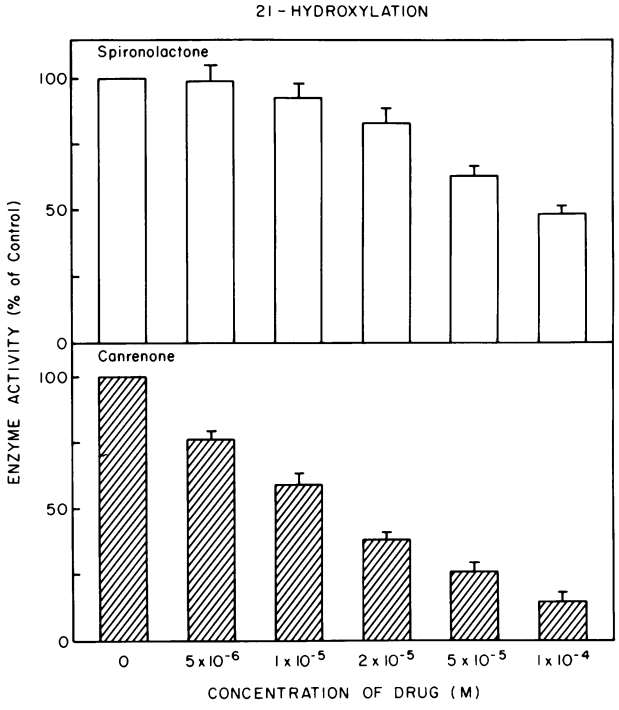


FIGURE 5. Effects of spironolactone and canrenone *in vitro* on 21-hydroxylase activity in guinea pig adrenal microsomes. See Greiner et al. (*16*) for experimental details.

Table 3. Effects of spironolactone or canrenone administration to guinea pigs on adrenal mitochondrial and microsomal cytochrome P-450 content.[a]

	Control	Spironolactone	Canrenone
Adrenal weight, mg/pair	446 ± 31	502 ± 33	472 ± 15
Protein, mg/g			
Mitochondrial	37.6 ± 1.2	36.3 ± 0.8	36.1 ± 0.8
Microsomal	48.6 ± 1.6	43.4 ± 1.2	43.0 ± 1.9
Cytochrome P-450, nmole/mg protein			
Mitochondrial	0.46 ± 0.03	0.38 ± 0.02[b]	0.45 ± 0.02[c]
Microsomal	1.99 ± 0.11	1.32 ± 0.12[b]	1.82 ± 0.10[c]

[a] For experimental details, see Greiner et al. (*16*).
[b] $p < 0.05$ (vs. controls)
[c] $p < 0.05$ (vs. spironolactone-treated)

converted by the adrenal cortex to a metabolite which promotes the destruction of adrenal cytochrome(s) P-450. In subsequent studies (*17*) it was found that only analogs of SL with the sulfur group at the C-7 position decrease cytochrome P-450 levels. Thus, SL appears to interfere with steroidogenesis through two related but distinct mechanisms: (1) SL and/or one or more of its metabolites compete(s) with normal steroid substrates for binding sites on cytochrome P-450, and (2) SL is converted to a toxic metabolite by adrenal enzymes, resulting in the destruction of cytochromes P-450 and loss of hydroxylase activity. The identity of the active metabolite is presently unknown and is now being pursued in several laboratories.

Another compound which requires conversion to an active metabolite before its toxic effects are manifested is carbon tetrachloride (CCl_4) (*18*). The hepatotoxic effects of CCl_4 are well known and have been extensively studied for many years. Less well known is that CCl_4 poisoning also produces adrenal necrosis in man as well as in some experimental animals (*19*). Until recently, little was known about the specific actions of CCl_4 on the adrenal cortex. When CCl_4 was given to guinea pigs and the animals killed 48 hr later, changes in adrenal microsomal steroid hydroxylases were found (Table 4). Both 17α- and 21-hydroxylase activities in ad enal microsomes were decreased by CCl_4 treatment but the mitochondrial 11β-hydroxylase was unaffected. Similarly, microsomal but not mitochondrial cytochrome P-450 levels were decreased by CCl_4.

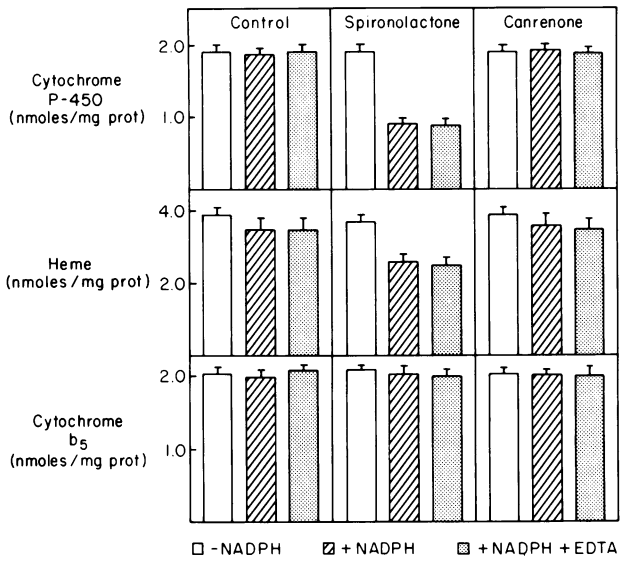


FIGURE 6. Effects of preincubation of guinea pig adrenal microsomes with spironolactone or canrenone on cytochrome P-450, heme, and cytochrome b_5 levels. See Greiner et al. (*16*) for experimental details.

It is reasonably well established that in the liver, the toxic effects of CCl_4 depend upon its conversion by cytochrome P-450-containing enzymes to the highly reactive trichloromethyl radical (*18*). However, the nature of the subsequent events in CCl_4 toxicity is somewhat controversial; the controversy is centered about the involvement of lipid peroxidation. The two prevalent hypotheses for the mechanism of action of CCl_4 include the conversion of CCl_4 to the trichloromethyl radical. However, one hypothesis advocates a direct attack of the radical on cellular macromolecules, including cytochromes P-450, resulting in the destruction of enzymes. The other hypothesis proposes an obligatory role for lipid peroxidation in the actions of CCl_4; that is, an attack on the unsaturated fatty acids of membrane phospholipids by the trichloromethyl radical, resulting in hydroperoxide formation and membrane disruption and secondarily causing a loss of cytochrome P-450 and associated enzyme activities.

In examining the effects of CCl_4 on adrenal steroidogenic enzymes, we attempted to determine if lipid peroxidation was involved. Lipid peroxidation activity was assayed as the formation of malon-

Table 4. Effects of carbon tetrachloride (CCl_4) administration to guinea pigs on adrenal microsomal and mitochondrial steroid hydroxylases.[a]

	Microsomes		Mitochondria	
	Control	CCl_4	Control	CCl_4
Protein, mg/g adrenal	41.7 ± 3.1	39.8 ± 4.1	34.2 ± 2.9	33.8 ± 2.8
Cytochrome P-450, nmole/mg protein	1.8 ± 0.2	0.8 ± 0.2*	0.5 ± 0.1	0.5 ± 0.1
17α-Hydroxylation, nmole/min/mg protein	7.6 ± 0.6	4.1 ± 0.5*	-	-
21-Hydroxylation, nmole/min/mg protein	2.9 ± 0.2	1.7 ± 0.2*	-	-
11β-Hydroxylation, nmole/min/mg protein	-	-	2.4 ± 0.2	2.5 ± 0.2

[a] CCl_4 was given to adult male guinea pigs as a single intraperitoneal injection 48 hr prior to sacrifice at a dose of 0.4 ml/kg body weight.
* $p < 0.05$ (vs. controls)

aldehyde, one of the breakdown products of hydroperoxides. In several preliminary experiments we found that the effects of CCl_4 on adrenal microsomal enzymes were readily demonstrable *in vitro* (Fig. 7). When CCl_4 was incubated with adrenal microsomes plus NADPH, a decline in the rates of steroid 17α- and 21-hydroxylation resulted. The decline in enzyme activity was dependent upon the duration of incubation and required the presence of NADPH, suggesting that CCl_4 was converted to an active metabolite by adrenal microsomes. Incubation with CCl_4 plus NADPH also produced a decrease in adrenal microsomal cytochrome P-450 content and, as previously found in the liver, CCl_4 plus NADPH stimulated lipid peroxidation in adrenal microsomes.

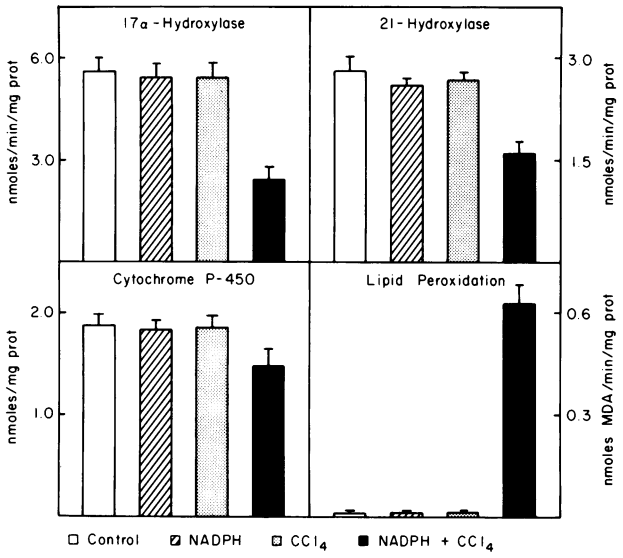


FIGURE 7. Effects of preincubation of guinea pig adrenal microsomes with carbon tetrachloride (CCl_4) on steroid hydroxylation and lipid peroxidation. Microsomes (2.0 mg protein/ml) were incubated in 1.15% KCl-0.05*M* Tris-HCl (pH 7.4) for 60 min at 37°C under air. NADPH (0.4m*M*) and/or CCl_4 (5 μl/ml) were added prior to the start of incubation. Lipid peroxidation was measured as the rate of production of malonaldehyde (MDA).

To determine if lipid peroxidation was essential for the toxic effects of CCl_4 on adrenal steroidogenic enzymes, similar incubations were carried out in the presence and absence of EDTA, a well known inhibitor of lipid peroxidation (Fig. 8). The lipid peroxidation initiated by CCl_4 plus NADPH was almost completely inhibited when 1.0 mM EDTA was also present in the incubation medium. Nonetheless the decreased in 17α- and 21-hydroxylase activities produced by NADPH plus CCl_4, as well as the decline in cytochrome P-450 levels, were unaffected by the EDTA. The time course for the decreases in 17α- and 21-hydroxylase activities during incubation of adrenal microsomes with CCl_4 plus NADPH was also unaffected by EDTA (Fig. 9). These observations indicate that CCl_4 can indeed stimulate lipid peroxidation in adrenal microsomes but that the lipid peroxidation is not obligatory for the toxic effects of CCl_4 on adrenal microsomal enzymes.

Although we have not been able to demonstrate any cause and effect relationship between lipid peroxidation and CCl_4 toxicity, the significance of lipid peroxidation in relation to steroidogenic enzyme activity remains an interesting question. It is known that lipid peroxication has deleterious effects on the membranous components of cells (*18*) and steroid hydroxylases are membrane-bound enzymes. However, relatively little is known about lipid peroxidation in steroid-producing tissues. The relationship between lipid peroxidation and steroidogenesis may provide fertile ground for future research since some of the factors known to promote lipid peroxidation, such as ascorbate, iron, and NADPH, are found in relatively high concentrations in steroidogenic tissues like the adrenal cortex.

The compounds discussed thus far all exert effects on steroidogenesis as a result of actions on the cytochrome P-450 portion of steroid hydroxylases.

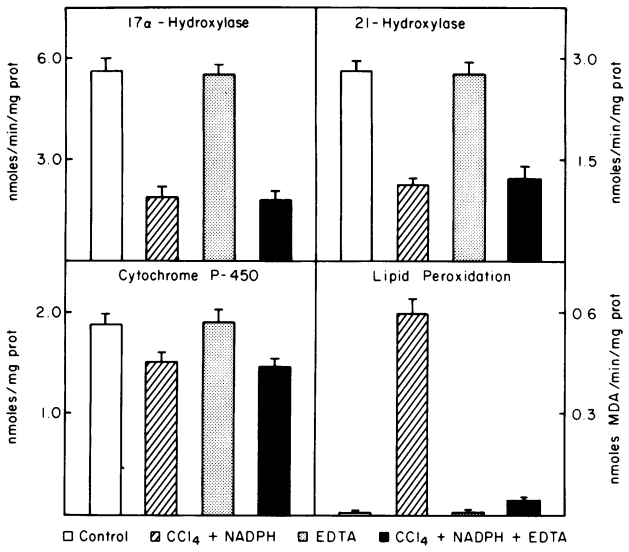


FIGURE 8. Effects of EDTA (1.0*mM*) on the carbon tetrachloride-induced changes in adrenal microsomal metabolism. Incubation conditions were as described for Fig. 7.

A number of heavy metals also inhibit steroid hydroxylation reactions but apparently via a different mechanism. Many metals including cadmium have been shown to exert inhibitory effects on hepatic cytochrome P-450-containing enzymes (*20*, *21*), and recently we found that cadmium administration to guinea pigs produced substantial decreases in adrenal steroid hydroxylases as well. The actions of cadmium on adrenal microsomal steroid metabolism are also demonstrable *in vitro*. Incubation of adrenal microsomes with Cd at 37°C produced concentration-dependent decreases in steroid 21-hydroxylase activity (Fig. 10). Similar changes in microsomal 17α-hydroxylase and mitochondrial 11β-hydroxylase activities were also observed. The decreases in 21-hydroxylase activity were proportional to the length of incubation and were temperature-dependent. Incubations at 0°C had no effects on enzyme activity.

The time-course for the changes in 21-hydroxylase activity in the presence of 50μM cadmium is shown in Figure 11. Incubation for 60 min produced almost complete loss of enzyme activity. Despite the large decrease in the rate of steroid metabolism, there was no change in cytochrome P-450 content in the microsomes. In addition, cadmium did not affect the interaction of steroid substrates with cytochromes P-450, as indicated by the absence of change in the size of the type I spectral change produced by 17α-hydroxyprogesterone, a substrate for 21-hydroxylation. However, cadmium produced decreases in NADPH-cytochrome P-450 reductase activity which were proportionately similar to the decreases in 21-hydroxylase activity. Thus, cadmium appears to exert its inhibitory effects on steroidogenesis via selective actions on the reduc-

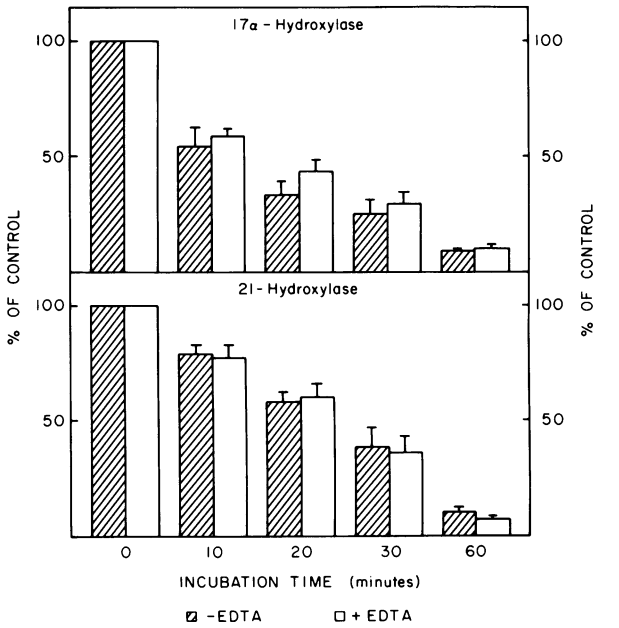


FIGURE 9. Time-course for the effects of carbon tetrachloride + NADPH on adrenal microsomal 17α- and 21-hydroxylase activities in the presence and absence of EDTA (1.0*mM*). Incubation conditions were as described for Figure 7.

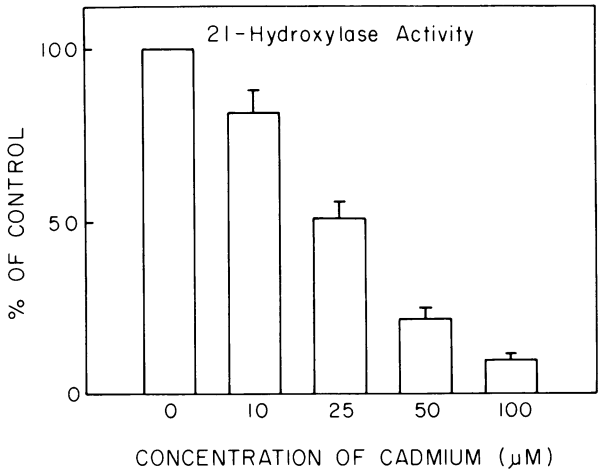


FIGURE 10. Effects of preincubation of guinea pig adrenal microsomes with cadmium on 21-hydroxylase activity. Microsomes (2.0 mg protein/ml) were incubated in 1.15% KCl-0.05*M* Tris-HCl (pH 7.4) for 30 min at 37°C under air in the presence of varying concentrations of cadmium chloride. After incubation, 21-hydroxylase activity was assayed as the rate of conversion of 17-hydroxyprogesterone to 11-deoxycortisol.

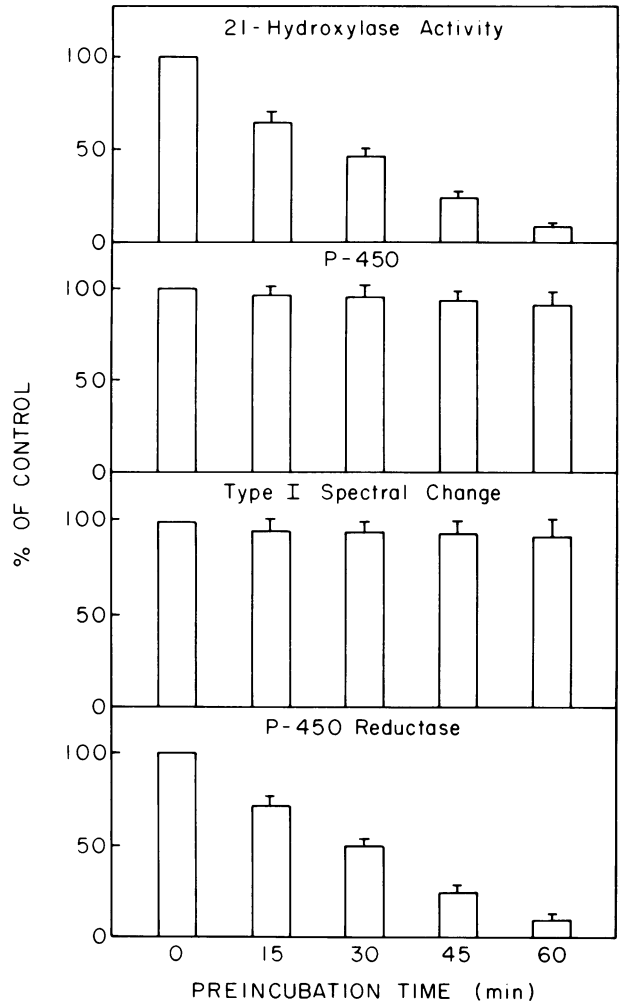


FIGURE 11. Time-course for the changes in adrenal steroid metabolism resulting from the incubation of microsomes with cadmium (50μM). Incubation conditions were as described for Fig. 10. Cytochrome P-450 was measured as the dithionite reduced CO complex and NADPH-cytochrome P-450 reductase activity was determined in the presence of 17-hydroxyprogesterone ($5 \times 10^{-5} M$), a substrate for 21-hydroxylation. The type I spectral change was obtained by addition of $5 \times 10^{-5} M$ 17-hydroxyprogesterone to adrenal microsomal suspensions.

tase portion of the steroid hydroxylase and not on the cytochrome P-450. Preliminary studies indicate that other metals such as zinc and lead act in a similar manner.

The compounds which have been discussed in this presentation are but a few of the many chemicals that can affect steroidogenesis. The toxicological significance of these agents as well as definitive mechanisms of action have in most cases yet to be established but represent areas of ongoing investigation in a number of laboratories. However, it should be borne in mind that there also remain many gaps in our knowledge of the fundamental mechanisms in the regulation of steroid hormone production. Only with a more complete understanding of the processes involved in steroidogenesis are we likely to be able to fully comprehend the actions of chemicals on this important metabolic pathway.

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